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PURIFICATION AND CHARACTERIZATION OF RECOMBINANT VACCINIA L1R PROTEIN FROM *ESCHERICHIA COLI*

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14. ABSTRACT:

The Vaccinia virus is closely related to the causative agent of smallpox and as such, is frequently used as a surrogate for smallpox in research and development efforts. The Vaccinia surface protein, L1R, is critical for viral entry into host cells, and because of its close similarity to its smallpox counterpart, it is a logical target for therapeutic and vaccine development. In this report, we describe a procedure and the typical outcome for the recombinant expression and purification of the L1R protein in *Escherichia coli* cells. L1R is solubilized and refolded from inclusion bodies after induction in *E. coli*, and its purification proceeds through two rounds of immobilized metal affinity chromatography, followed by size-exclusion chromatography. The typical yield is approximately 2 mg/L of bacterial culture. The recombinant L1R was shown to bind to a known anti-L1R antibody, which indicated that the protein was intact and properly folded. This procedure allows for the in-house production of L1R for research efforts such as antibody discovery and immunoassay development.

15. SUBJECT TERMS

Vaccinia virus (VACV)	Smallpox	Recombinant	Protein expression
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PREFACE

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1. INTRODUCTION

1.1 Background

Vaccinia virus (VACV) is the active component of the vaccine that was used to eradicate smallpox. Although the World Health Organization declared that smallpox was eradicated in 1979, the potential use of the smallpox virus as a biological weapon still exists, which would have particularly devastating consequences because routine vaccination against smallpox was discontinued in 1972. Because of its close relation to smallpox, VACV is frequently used as a model for the smallpox virus in laboratory research, and its proteins are used as surrogates for smallpox proteins.

1.2 Objective

The objective of this study was to characterize the preparation of the recombinant VACV L1R protein fragment by denaturing, refolding, and purifying material expressed into inclusion bodies in *Escherichia coli*.

1.3 VACV L1R Protein

VACV L1R is a conserved component of the viral envelope that plays a major role in viral entry at the host cell surface (Ravanello and Hruby, 1994). Because of its surface exposure and importance in the virus life cycle, the L1R protein is an antigen of interest when creating neutralizing affinity reagents against VACV and for creating novel formulations of the smallpox vaccine (Kaever et al., 2014; Walper et al., 2014).

VACV L1R is a 285 residue protein, with the C-terminal 100 amino acids embedded in the viral membrane. The N-terminal portion of the protein, which is expressed at the surface of the virion, folds as a bundle of helices surrounding a pair of beta strands (Su et al., 2005). This N-terminal region is nearly identical to its smallpox and monkeypox counterparts, which contain only one and two amino acid differences, respectively.

In this report, we describe the procedure for recombinant VACV L1R purification from *E. coli* and its typical yield. The purification procedure was adapted from previous reports (Su et al., 2005) with some modifications. This procedure and the results will be useful to other researchers who wish to produce L1R in-house for further research, such as antibody discovery or immunoassay development.

2. MATERIALS AND METHODS

2.1 Vector Construction

An open reading frame (ORF) containing residues 1 through 185 of the VACV L1R protein was amplified from genomic Vaccinia DNA using high-fidelity PfuTurbo DNA polymerase (Agilent Technologies, Santa Clara, CA). Subsequently, the ORF was subcloned into pET21b using the *Nde1* and *Xho1* restriction sites, which resulted in the addition of a C-terminal hexahistidine tag. This plasmid was sequence-verified. While this plasmid sequence differs from the published structure, this mutation appears in several Vaccinia and Variola sequences in the National Center for Biotechnology Information (Bethesda, MD) databases.

2.2 Recombinant Protein Expression in *E. coli*

BL21 DE3 cells were transformed with the pET21b:L1R plasmid and spread onto lysogeny broth (LB) agar plates containing ampicillin (working concentration of 100 μg/mL). A single colony was selected and cultured in LB plus ampicillin media, from which a glycerol freezer stock was prepared. Expression starter cultures in 5 mL LB plus ampicillin were then inoculated with this freezer stock.

From the overnight starter culture, 4 L flasks containing 1 L of LB plus ampicillin were inoculated. These cultures were grown at 37 °C with vigorous shaking until they reached an optical density (OD) of 1.0 at 600 nm. At this point, they were induced by the addition of isopropyl-ß-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After induction, the cultures were grown at 22 °C for 18 h.

Cells were harvested using centrifugation at 6000×*g*. Pellets can be stored at –20 °C for at least a year before performing protein extraction. Harvested cells were resuspended in lysis buffer consisting of 100 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 5 mM $MgCl_2$, 0.02 mg/mL lysozyme, 0.02 mg/mL DNase, and 1 mM phenylmethylsulfonyl fluoride at 10 mL/g of cells. After lysis by sonication, additional DNAse was added, and the sample was incubated at room temperature for 10 min. Alternatively, 4 cycles of alternate freezing and thawing of the pellet showed sufficient cell lysis to extract L1R, although the yields were slightly lower.

2.3 Inclusion Body Enrichment and Solubilization

After lysis was performed, the preparation was enriched for inclusion bodies by 3 cycles of centrifugation (10,000×*g* for 15 min), the supernatant was decanted, and the pellet was resuspended in lysis buffer (~2 min of sonication with a 50% duty cycle was used to aid in resuspension). This procedure removed soluble components of the bacterial lysate, leaving behind the rapidly sedimenting inclusion bodies and other insoluble cellular components. A final resuspension was performed in the detergent-free buffer (100 mM Tris HCl, pH 8.0, and 150 mM NaCl), and then a final centrifugation step was performed. After the supernatant was decanted, the pellet was dissolved in approximately 6× the pellet volume of denaturing buffer A (100 mM Tris HCl, pH 8.0, 6 M guanidine HCl, 300 mM NaCl, 2 mM dithiothreitol [DTT], and

10 mM imidazole). In addition, we had success in freezing the inclusion body pellet at –20 °C after decanting the supernatant. The pellets may be frozen for up to 2 weeks with no apparent adverse effects, and it is reasonable to expect that they could also be frozen for longer periods or at –80 °C.

Mechanical disruption with a pipettor and end-over-end mixing for 30 min were required to fully dissolve the pellet in denaturing buffer A. To ensure complete dissolution, an additional (~1×) volume of denaturing buffer A was added after no further visible clearing of the pellet was observed. This solution was cleared by centrifugation at 6000×*g* for 20 min to separate any materials that had not dissolved.

2.4 Denaturing Chromatography (Purification Step 1)

The supernatant was decanted into a beaker and incubated with 6 mL of nickel-charged, immobilized-metal-affinity chromatography (IMAC) resin, which had been pre-equilibrated in denaturing buffer A. After 20 min of gentle stirring to load any His-tagged material onto the resin, the resin was captured on a fritted chromatography column, washed with 36 mL of denaturing buffer A, and eluted with 12 mL of denaturing buffer B (100 mM Tris HCl, pH 8.0, 6 M guanidine HCl, 300 mM NaCl, 2 mM DTT, and 400 mM imidazole).

2.5 Refolding Method

The elution from Purification Step 1 was immediately poured into a beaker containing 240 mL (~20× elution volume) of freshly made and cold refolding buffer (100 mM Tris HCl, pH 8.0, 400 mM L-arginine HCl, 5 mM reduced L-glutathione HCl, and 0.5 mM oxidized L-glutathione HCl) while the buffer was being magnetically stirred. The stirring was reduced to a gentle mixing, and the beaker was covered and left at 4°C for 24 h. After 24 h, the refolding solution was dialyzed against 4 L of post-refolding dialysis buffer (100 mM Tris HCl, pH 8.0, and 150 mM NaCl) for a total of 4 h with one buffer change at 2 h.

2.6 Concentration of His-Tagged Material (Purification Step 2)

Following refolding and dialysis, the large volume of highly dilute protein was concentrated using IMAC chromatography. The protein solution was pumped across a 5 mL His-Trap HP column using an AKTA (Chicago, IL) fast protein liquid chromatography system. Elution proceeded with a steep (for 30 min at 3 mL/min) imidazole gradient from native buffer A (10 mM Tris HCl, pH 8.0, 150 mM NaCl, and 10 mM imidazole) to 100% native buffer B (10 mM Tris HCl, pH 8.0, 150 mM NaCl, and 400 mM imidazole).

2.7 Size-Exclusion Chromatography ([SEC] Optional Purification Step 3)

Denaturing IMAC chromatography generally yields relatively pure protein, but depending on the intended use of the material, SEC may be necessary to remove misfolded or multimerized L1R. We elected to perform this step. Elutions from Purification Step 2 were collected and applied to a Superdex S200 column (GE Healthcare Life Sciences,

Pittsburgh, PA), which was pre-equilibrated using SEC buffer (10 mM Tris HCl, pH 8.0, and 150 mM NaCl), and then, the elutions were isocratically eluted. Two peaks were evident: one corresponded to the expected molecular weight of L1R (~24 kDa) and the other, larger peak, which was based on protein gel analysis, was likely to be misfolded or aggregated L1R. The peaks corresponding to L1R of the expected molecular weight were pooled, aliquoted, and snap-frozen in liquid nitrogen before being stored at –80 °C.

2.8 Electrophoresis

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method was performed on protein samples using NuPAGE 4–12% Bis-Tris 1.0 mm protein gels (LifeTechnologies [a retired brand of Thermo Fisher Scientific, Inc.], Waltham, MA) and NuPAGE lithium dodecyl sulfate sample buffer (LifeTechnologies) under reducing conditions in accordance with the manufacturer's instructions.

2.9 Protein Concentration Determination

Recombinant L1R lacks tryptophan residues and as such, has a low ε_{280}, which makes the determination of spectrophotometric concentration unreliable. Consequently, we employed the colorimetric Advanced Protein Assay (Cytoskeleton, Inc., Denver, CO) using known concentrations of bovine serum album to generate a standard curve from which the concentration of L1R was determined. This was conducted in accordance with the manufacturer's instructions.

2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

Each L1R preparation was screened with sandwich ELISA using the 7D11 anti-poxvirus L1 antibody to capture L1R and an anti-His HRP antibody to detect the His-tagged L1R. The 7D11 antibody was coated at 10 µg/mL and incubated overnight at 4 °C. All subsequent incubations were at 37 °C for 1 h, and the plates were washed 3 times with phosphate buffered saline (PBS) containing 0.1% Tween after each step. After coating, each plate was blocked with 5% milk in PBS, incubated with the L1R preparations at 100 µg/mL, and then, the Invitrogen anti-His horseradish peroxidase was used at 10 µg/mL before development with HRP substrate ABTS.[1] The plate absorbance was read at 405 nm for 10 min after substrate was added. Controls to check for nonspecific binding were added by omitting L1R from the sandwich.

3. RESULTS

3.1 Vector Construction

Genomic DNA from VACV was obtained through the Critical Reagent Program, from which the ORF of amino acids 1–185 of the L1R protein was PCR[2] amplified and subcloned into the pET21b plasmid, which added a six histadine tag to the expressed protein at

[1] ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid).
[2] PCR: polymerase chain reaction.

the C-terminus. The construct was then sequenced to verify that it matched the L1R found in literature.

3.2 Protein Purification and Yields

The *E. coli* BL21 DE3 cells that were transformed with the pET21b:L1R plasmid were grown in 1 L of LB plus ampicillin to an OD of approximately 1.0 at 600 nm. Protein expression was then induced by adding 1 mM of IPTG. Induction continued for 18 h at room temperature, after which the cells were harvested and L1R was purified as described Section 2.

Using SDS-PAGE after Purification Step 2, several species were observed in the sample consisting of different molecular weights. Because the sizes of these proteins appeared to be multimers of L1R, we surmised that the higher molecular weight species were L1R polypeptides that were covalently linked by a disulfide bond. To separate L1R monomers from higher-order species, we performed SEC (Figure 1).

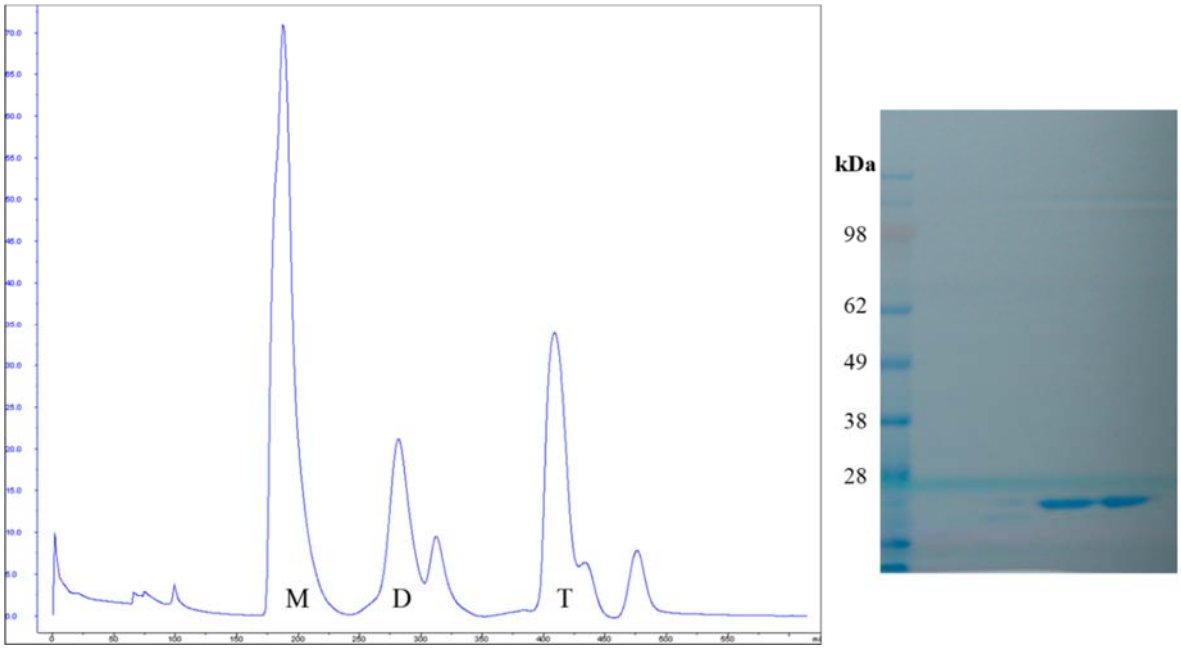

Figure 1. (Left) Chromatogram of VACV L1R elutions from SEC (the peaks are L1R monomer [M], dimer [D], and trimer [T]). (Right) The SDS-PAGE image shows representative fractions from the monomer elution peak, along with a molecular weight standard for reference. L1R runs just below the 28 kDa marker, as expected. The molecular weight of recombinant L1R is 20.7 kDa, based on the amino acid composition.

The concentration of pooled monomer SEC fractions was determined using a colorimetric assay because of the poor absorbance of light by L1R at 280 nm. The final yield of L1R monomers from 1 L of bacterial cell culture was approximately 2.2 mg.

3.3 Protein Verification Using ELISA

To verify that the recombinantly produced L1R was the correct, properly folded protein, we performed an ELISA screening to determine whether 7D11, an antibody known to bind L1R, could recognize the protein. The structural basis of 7D11 binding to L1R has been extensively characterized previously (Su et al., 2007).

The ELISA revealed that 7D11 bound effectively to two separate preparations of L1R (Figure 2). Discrepancies between the strength of binding to each of the preparations was likely due to the age of the protein, as one preparation was more than a year older than the other, and it is possible that older protein may have degraded.

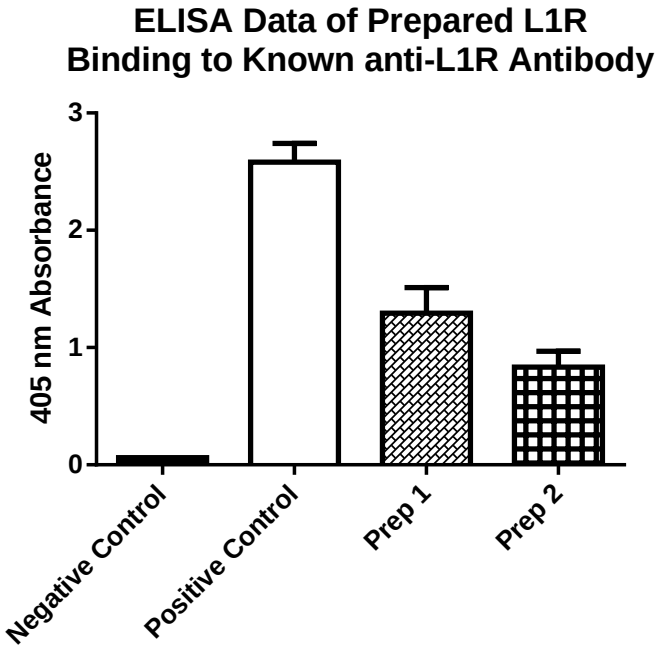

Figure 2. ELISA data showing the binding of a known antibody against L1R (7D11) to two separate preparations of recombinant L1R. Both preparations were recognized by 7D11, which indicated that the protein was intact and properly folded. The signal of the positive control was designed to saturate the response and as such, showed a much higher absorbance at 405 nm than did our preparations.

4. DISCUSSION

The methodology described in this study details the recombinant expression and purification of intact, properly folded VACV L1R in *E. coli*. L1R was expressed in *E. coli* inclusion bodies, and its purification was performed by solubilizing and refolding the material from the insoluble portion of cell lysate. Two rounds of IMAC purified His_6-tagged L1R from other inclusion body components, and a final round of SEC purified L1R monomers from higher-order multimers that formed because of the refolding process. Approximately 2 mg of purified L1R were produced from 1 L of *E. coli* cell culture. Proper folding of the protein was verified by assessing the binding of a known anti-L1R antibody to two separate preparations of L1R using ELISA. Using this procedure, researchers can generate VACV L1R in-house to support research programs such as antibody discovery or immunoassay development.

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ACRONYMS AND ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
HRP	horseradish peroxidase
IMAC	immobilized-metal-affinity chromatography
IPTG	isopropyl-ß-D-thiogalactopyranoside
LB	lysogeny broth
OD	optical density
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
VACV	Vaccinia virus

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